CHEMORECEPTION OF MARINE CHEMICAL DEFENSES

A Thesis
Presented to
The Academic Faculty

by

Zinka Bartolek

In Partial Fulfillment
of the Requirements for the Degree
BS in Biomedical Engineering in the
School of Engineering

Georgia Institute of Technology
December 2017

CHEMORECEPTION OF MARINE CHEMICAL DEFENSES

Approved by:

Dr. Julia Kubanek
School of Biological Sciences
Georgia Institute of Technology

Dr. Lin Jiang
School of Biological Sciences
Georgia Institute of Technology

Dr. Esfandiar Behravesh
Wallace H. Coulter Department of Biomedical Engineering
Georgia Institute of Technology

Date Approved: 12 Dec 2017

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

This research is supported by National Science Foundation collaborative research (Grant No. IOS1354837) and President's Undergraduate Research Salary Award (PURA) to Zinka Bartolek.

LIST OF FIGURES

SUMMARY

Many marine organisms protect themselves from predators by concentrating triterpene glycosides in their tissue. It has been proposed that predatory fish response to triterpene glycoside formoside is mediated by chemoreceptors. RL-TGR, a co-receptor involved in triterpene glycoside signaling has been identified in zebrafish. However, the mechanism and scope of function of RL-TGR is not fully understood. Formoside form marine sponge *E. formosus* and thornasteroside A from sea star *A. Planci* were purified using reversed phase HPLC, and the structure characterized via ^{1}H NMR spectroscopy. To test for a functional response, RL-TGR was expressed in HEK 293 cells along with β_2AR, which was necessary for trafficking to the cell membrane. HEK 293 cells expressing RL-TGR were treated with formoside and thornasteroside A and the activity of RL-TGR was determined by measuring the changes in the cAMP levels in these cells. This study found no significant effect of either formoside or thornasteroside A on the cAMP production in cells expressing RL-TGR. Therefore, a functional response of RL-TGR to formoside and thornasteroside A was not observed in this study. Understanding the scope of chemical defenses and chemoreception in marine environments is crucial to expanding our knowledge of the structure and function of marine communities.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Many sessile and soft-bodied marine organisms use chemical defenses to protect from predation, colonization by bacteria and overgrowth by neighboring organisms (Paul et al., 2006). For example, the evolutionary success of soft corals in areas of high levels of predation has been attributed to their production of large amounts of chemical defense compounds in their tissue (Blunt et al., 2007). Chemical defense compounds are secondary metabolites produced by either the organism itself, a bacterial symbiont, or are sequestered from another species (Moore at al., 2006). Some tropical sponges exhibit wound activated chemical defenses, where tissue damage activates a rapid conversion of precursor molecules to defensive compounds (Thoms et al., 2008). Other marine sponges such as *Erylus formosus* protect themselves from predators by concentrating aversive secondary metabolites such as formoside and other triterpene glycosides in their tissue (Kubanek et al., 2002).

Triterpene glycosides are also found in other soft-bodied marine organisms, such as sea stars and sea cucumbers (Pawlik et al. 1995). Tropical sea stars *Acanthaster planci* and *Linckia laevigata* have unique and complex surface microtopographies that were proposed to physically deter the settlement of fouling organisms (Guenther et al., 2007). Research showed, however, that surface microtopographies of sea stars alone were not effective in preventing biofouling (Gunther et al., 2007). This suggests that sea stars have developed alternative methods for protection against the settlement and growth of fouling organisms. Triterpene glycosides thornasteroside A and nodososide have been isolated

from sea stars *A. planci and L. laevigata* (Minale et al., 1983 and Kitagawa et al., 1978). It is proposed that these aversive compounds are responsible for the protection of sea stars form both predation and biofouling (Minale et al., 2012).

It has been shown that live zebrafish reject foods laced with sponge triterpene glycosides including the compound formoside found in sponge *E. formosus* (Cohen et al., 2008). Research has proposed that the predatory fish response to triterpene glycosides is mediated by chemoreceptors (Kubanek et al., 2000). However, very little is known about the molecular pathways that control responses to chemical defenses in predatory fish.

Recently, a co-receptor involved in triterpene glycoside signaling in zebrafish has been identified. This receptor, RAMP-like triterpene glycoside receptor (RL-TGR), is expressed in zebrafish head sections, which suggests it is involved in a gustatory response to aversive compounds found in prey (Cohen et al., 2010). However, the exact structure and function of RL-TGR is not known. It has been proposed that RL-TGR forms a signaling complex with a G-protein coupled receptor (GPCR) to detect triterpene glycosides (Cohen et al., 2010). Previous studies suggest that complexing with a GPCR is necessary for both the trafficking of RL-TGR as well as the functional response to a ligand (Cohen et al., 2010).

It has been shown that RL-TGR responds specifically to triterpene glycoside chemical defenses in marine sponges derived from terpenoid pathways such as formoside from *E. formosus* and a mixture of ectyoplasides A and B from *Ectyoplasia ferox* (Cohen et al., 2010). Aversive compounds such as capsaicin and sceptrin produced by non-terpenoid biosynthetic pathways did not stimulate this receptor (Cohen et al., 2008).

However, not all terpene glycosides activated RL-TGR, for example, plant cardiac glycosides ouabain and digoxin showed no effect on RL-TGR (Cohen et al., 2010).

To further our understanding of chemoreception, it is important to determine the scope of RL-TGR and triterpene glycoside based defenses. The goal of this project is to determine whether RL-TGR responds to triterpene glycosides isolated from sources other than marine sponges, such as thornasteroside A from *A. planci*. To achieve these goals, thornasteroside A will be isolated from sea star *A. planci*. Afterwards, the response of RL-TGR towards this compound will be recorded by measuring cAMP levels in human embryonic kidney (HEK293) cells transfected with mammalian expression vectors containing wild-type RL-TGR and an external GPCR, beta-adrenergic receptor (β_2AR).

Chemical defenses are used by a variety of marine organisms and shape the structure of marine ecosystems by driving feeding behavior, organization of communities and speciation (Lunceford 2015). Investigating how marine organisms use chemical defenses to interact with their environment is crucial to understanding predator prey interactions. The further study of the scope and function of RL-TGR will add to the knowledge of molecular mechanisms of chemical defenses. This will contribute to the understanding of predator-prey interactions and feeding patterns in marine environments. Further research of chemical defenses and chemoreception will help us understand both chemical and behavioral ecology of marine environments.

CHAPTER 2

METHODS AND MATERIALS

Solid Phase Extraction (SPE)

Samples of *E. formosus* and *A. planci* were previously collected in Fiji, frozen, lyophilized and extracted with methanol to produce a crude extract. The SUPLECO Envi-18, 10g, 60mL column was used for separation of crude extracts. Crude extracts *from E .formosus* and *A. planci* (0.5g each) were dissolved in methanol, mixed with 0.5g of Envi-18 column packing and dried. After conditioning the columns, the crude extracts combined with column packing were placed on top the columns and covered with glass wool. The total eluent volume was 50ml, with the first eluent being 100% water, followed by a 10% increase in methanol until the final eluent was 100% methanol.

Thin Layer Chromatography (TLC)

Normal phase TLC was used to analyze the SPE fraction collected from *E. formosus* and *A. planci* crude extracts. A silica plate was used with the mobile phase consisting of n-butanol, acetic acid and distilled water in a ratio of 3:1:1. All TLC plates were stained with 5% sulfuric acid in ethanol.

High performance liquid chromatography (HPLC)

a) Formoside method:

Reversed phase HPLC (dual wavelength) was used to purify formoside from extracts of *E. formosus*. The sample was dissolved in 90% methanol and 10% distilled water to make an 8mg/1mL solution. The solution was filtered with a 0.22 μm filter. An isocratic method with 90% methanol and 10% distilled water (0.1% trifluoroacetic acid added to the water) was used. The C18 semi-prep column (250 mm ×10 mmm) was used with 500 μL injections, a wavelength of 210 nm and a flow rate of 3 ml/min.

b) Thornasteroside method:

Fractions 6 and 7 from SPE separation of *A. planci* crude extract were found to contain thornasteroside A. These two fractions were combined. To further purify thornasteroside A, reversed-phase HPLC with UV detection (210nm wavelength) was done with this sample. For method development, an analytical Altima C18 250mm x 4.6mm column was used. The sample was dissolved in methanol to a concentration of 10mg/ml, and a volume of 5μl was used for each injection. The final method for separation consisted of a mobile phase gradient from a 5% acetonitrile, 95% water solution to a 53% acetonitrile, 47% water solution in 15min followed by a 5 min gradient of a 53% acetonitrile, 47% water solution to a 100% acetonitrile solution, with a flow rate of 1ml/min. Once this method was developed, a semi-preparative Altima C18 250mm x 10.0mm column was used to collect larger quantities of eluting molecules. The injection volume was increased to 100μl and the flow rate was increased to 3ml/min.

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR)

NMR was used to confirm the structure and purity of formoside isolated from sponge *E. formosus* and thornasteroside A isolated from sea star *A. planci*. Sample was dissolved in deuterated methanol and ^{1}H NMR spectrum with 64 scans was acquired with a Bruker AVII-500 MHz instrument.

Transfection Protocol

The TurboFect Transfection Reagent protocol was used to transfect HEK293 cells with wild type RL-TGR and β_2AR. Twenty four hours prior to the transfection, HEK293 cells were plated in 12-well plates containing sterilized cover slips at a density of 1×10^5 cells/well. Four different experimental groups were used in the transfection: a negative control group transfecting the empty pcDNA 3.1 vector, a negative control group transfecting wild type RL-TGR without β_2AR to account for trafficking of RL-TGR to the cell membrane, an experimental group transfecting wild type RL-TGR and β_2AR in a ration of 3:1, and an experimental group transfecting wild-type RL-TGR and β_2AR in a ration of 1.5:1. The recommended amounts of DNA in the protocol were followed, however, in both experimental groups the total DNA amount of 2μg included both the wild type RL-TGR and β_2AR.

Immunofluorescence Protocol

The immunofluorescence protocol was performed 48 h after the transfection protocol to confirm the expression of wild type RL-TGR in the membrane of HEK293 cells. Media was aspirated from the wells containing transfected cells on cover slips. 1

mL of chilled 4% paraformaldehyde was added to fix and permeabilize the cells. The cells were incubated for 10 min at room temperature. Paraformaldehyde was removed and 1 mL of blocking buffer (PBTr (PBS + 0.3% TritonX) + 4% bovine serum albumin (BSA)) was added and incubated for 5 min at room temperature. Cells were stained with two different primary antibodies, a α-RL-TGR rabbit antibody, and a α-Strep II mouse antibody, since the RL-TGR sequence used in this experiment contained a Strep II tag. The primary antibodies were diluted 1:1000 in PBTr + 4% BSA, and spun for 5 min at 14000 rpm. Parafilm was added to the bottom of a 150 mm Petri dish, and 50 μL of the combined primary antibodies was added to the parafilm. Individual cover slips with cells were picked up from the 12-well plate and placed cell side down onto the antibody. The cells were incubated for 1h with the primary antibodies at room temperature. Cover slips were transferred back to the 12 well plate cell side up and washed 3 × 5 min with PBS. The corresponding secondary antibodies (goat α-rabbit Alexa 488, and goat α-mouse Dylight 550) were diluted in PBS 1:1000 and spun for 5 min at 14000 rpm. 50μL of the combined secondary antibodies was added to the parafilm in the Petri dish and cover slips were placed cell side down onto the secondary antibodies. Cells were incubated for 1h with the secondary antibodies at room temperature. Cover slips were transferred back to the 12-well plate and washed 3X 5 min with PBS. Cover slips were mounted onto glass slides with 1μL of DAPI in Vectashield and sealed with nail polish. Results were visualized with the Zeiss 700 Confocal microscope.

cAMP Assay Protocol

The cAMP assay was performed according to the protocol described by the Promega cAMP-Glo Assay manual. Twenty four hours prior to the cAMP assay, transfected HEK 293 cells were plated into 384 well plates at a concentration of 5000 cells/well. Before proceeding with the assay, cells were treated with 5μM and 10μM concentrations of formoside and thornasteroside A for 15min. After treatment, cells were lysed and the cAMP level in the cells was detected and correlated to luminescence as stated in the protocol. Luminescence was measured with a plate-reading luminometer.

CHAPTER 3

RESULTS AND DISCUSSION

Purification of triterpene glycosides from marine organisms

1. Purification of formoside from marine sponge E. formosus

Formoside was purified from extracts of sponge *E. formosus* using reversed phase HPLC as shown in Figure 1 using the method described earlier. The peak between 22 min and 28 min was found to contain formoside by TLC and ^{1}H NMR spectroscopy. The additional peak shoulders at 23min and 28 min indicate that the peak representing formoside contains impurities. Since the impurities could not be eliminated by different HPLC methods, formoside was collected only from 24 min to 26 min to minimize contamination.

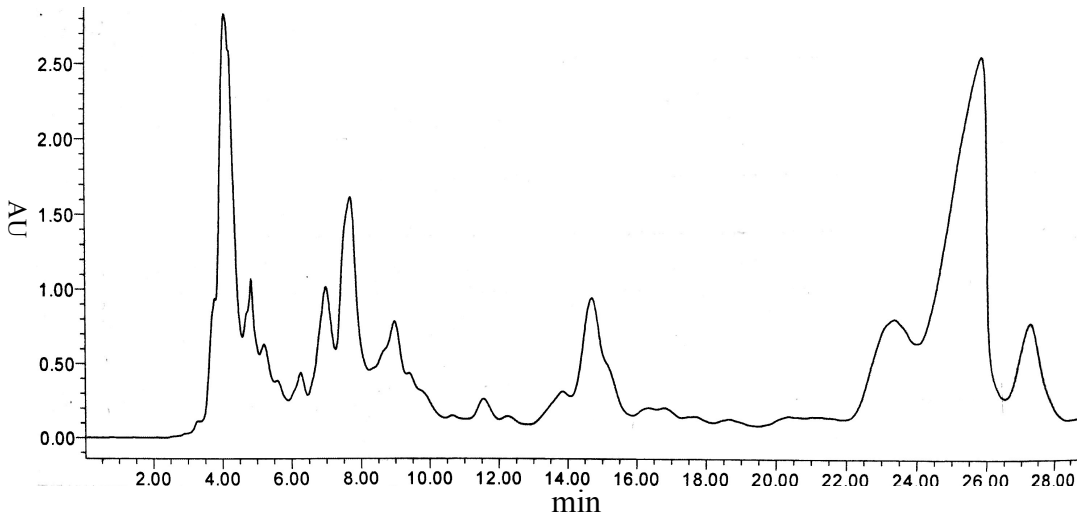

Figure 1. Reverser phase HPLC spectrum of *E. formosus* extract. Formoside was identified to elute between 22 min and 28 min (λ = 210nm).

The existence and purity of formoside collected was determined by ^{1}H NMR spectroscopy. The NMR spectrum of collected formoside (Figure 2) was compared with a standard spectrum of pure formoside found in literature. The purity of formoside was determined to be ~90% by peak integration.

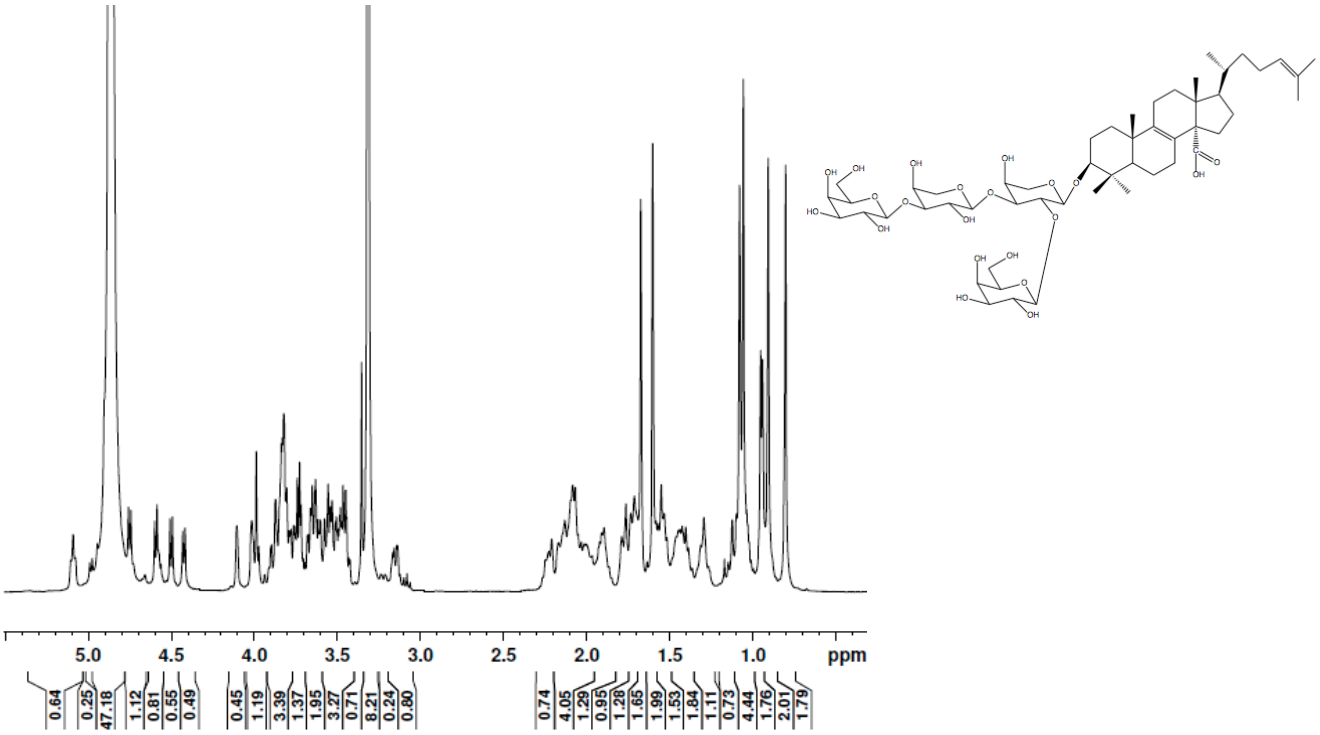

Figure 2. ^{1}H NMR spectrum of formoside. Left: ^{1}H NMR spectrum of formoside collected from HPLC peak from 24 min to 26 min. Formoside was found to be around 90% pure. The integrated areas for each peak used in purity calculations can be seen bellow the ppm axis. Right: Structure of formoside.

These results confirm the existence and purity of formoside sufficient for further experiments. In total, 150 mg of formoside has been purified from extracts of *E. formosus.*

2. Purification of Thornasteroside A from A. planci

Eleven different SPE fractions of crude extract of *A. planci* were obtained by SPE separation method described earlier. These fractions were tested for the presence of triterpene glycosides, and in particular thornasteroside A. Normal phase TLC was used to test for the presence of thornasteroside A in different SPE fractions of *A. planci* and *L. laevigata* against formoside as a standard (Figure 3).

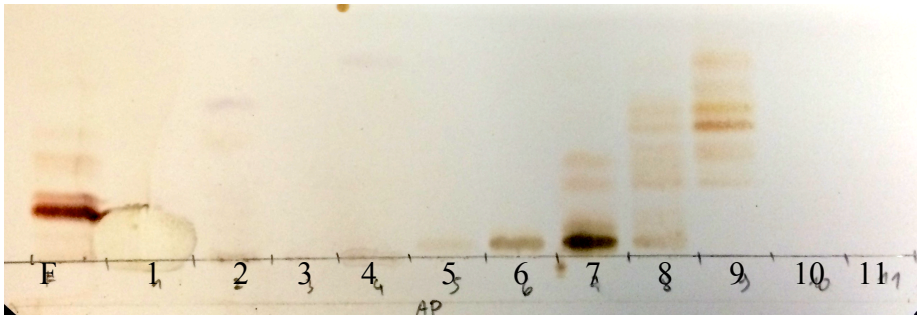

Figure 3. Normal phase TLC plates of SPE fractions of crude extracts of *A. planci*. The first column is formoside, followed by 11 different SPE fractions of *A. planci*.

Due to the varying polarities of formoside compared to thornasteroside A, Thornasteroside A was expected to have a lower R_f value compared to formoside. Therefore, SPE fractions 6, 7 and 8 of *A. planci* where hypothesized to contain thornasteroside A (Figure 3). As can be seen in Figure 3, the presence of multiple bands in fractions 6, 7 and 8 indicates that they contain many different compounds in addition to thornasteroside A. To further purify thornasteroside A from these unknown compounds, an HPLC method was developed (Figure 4). SPE fractions 6 and 7 from *A. planci* were combined for HPLC purification. The materials eluting at 5 min in Figure 4 included thornasteroside A according to mass spectrometry and ^{1}H NMR spectroscopy (Figure 5 and 6).

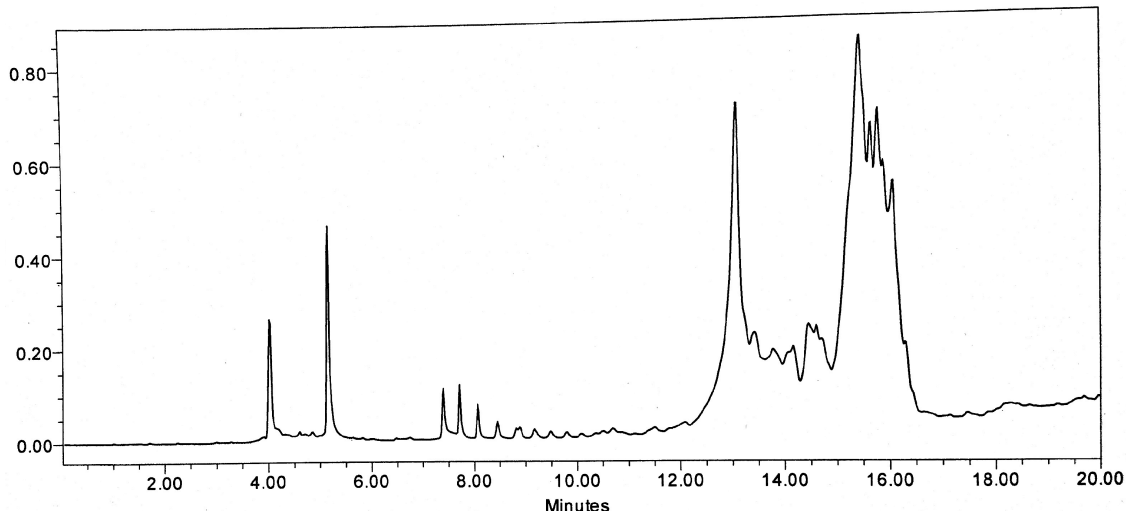

Figure 4. Reversed phase HPLC spectrum of *A. planci* extract. SPE fractions 6 and 7 of *A. planci* extract were combined for HPLC purification. Thornasteroside A was identified to elute at 5 min (λ = 210nm).

To confirm the structure and existence of thornasteroside A, ^{1}H NMR spectrum was obtained. As can be seen in Figure 5, the obtained spectrum is very similar to the pure thornasteroside A spectrum found in literature, with the exception of some small impurities.

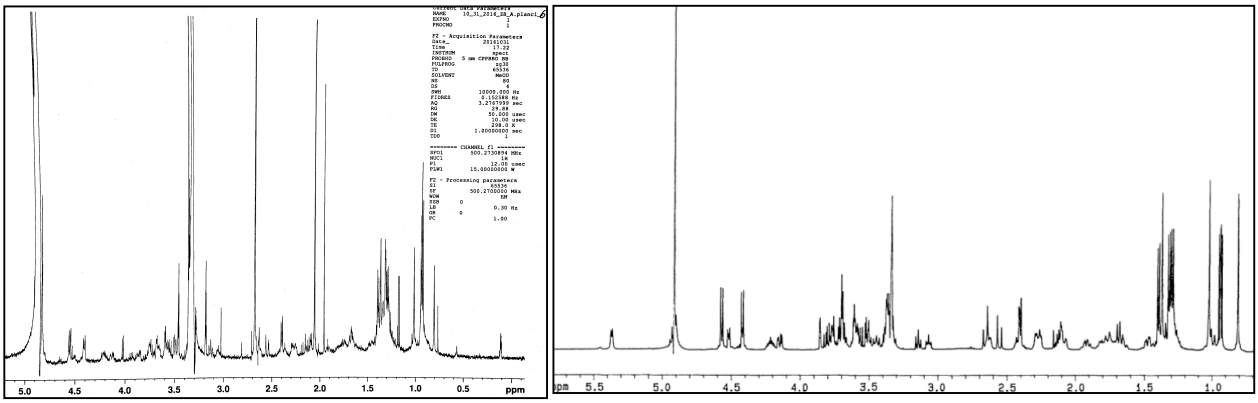

Figure 5: ^{1}H NMR spectra of thornasteroside A. Left: ^{1}H NMR spectra of thornasteroside A isolated from *A. planci*. Right: ^{1}H NMR spectra of pure thornasteroside A found in literature (Minale, 2012). In both cases the compound was dissolved in deuterated methanol.

High-resolution mass spectrometry data was obtained to confirm the presence of thornasteroside A in the sample. Negative ionization mode was used due to the presence of the sulfate group in the structure of the molecule (Figure 6). The theoretical mass of

thornasteroside A is known to be 1243.5423 from literature, while the observed mass was found to be 1243.5414 as can be seen in Figure 6. The percent error in ppm was determined to be 0.7, which suggests that the isolated compound is thornasteroside A. In total, 5 mg of thornasteroside A was isolated from extracts of *A. planci.*

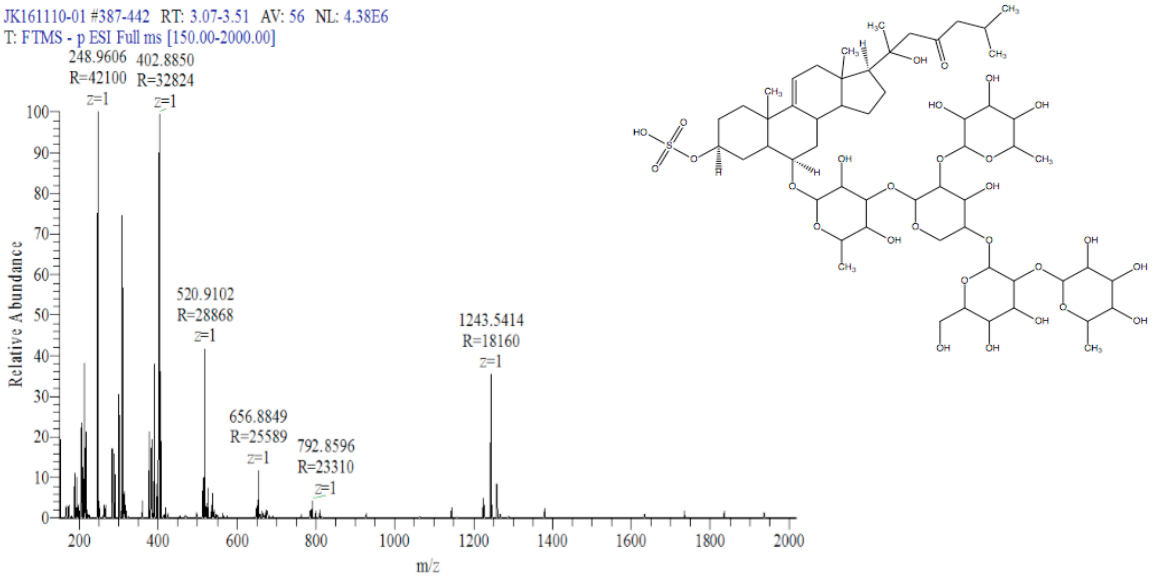

Figure 6: Mass spectrometry of thornasteroside A. High resolution mass spectrometry data of thornasteroside A in negative ionization mode (left) and the structure of thornasteroside A (right).

Expression of RL-TGR in HEK 293 cells

To be able to test the functional response of RL-TGR to triterpene glycoside chemical defenses, RL-TRG was expressed in HEK 293 cells along with β_2AR. HEK 293 cells were transfected as described in the methods and imaged via confocal microscopy. Transfected cells were stained for the presence of RL-TGR with a α-RL-TGR antibody Alexa 488 (green), and the nucleus was stained with DAPI in Vectashield (blue). As can be seen in Figure 7, cells transfected with both RL-TGR and β_2AR were found to express RL-TGR only on the cell membrane. In contrast, cells transfected with RL-TGR only showed expression of RL-TGR throughout the cell surface, without evidence of trafficking of RL-TGR to the cell membrane (Figure 7). The negative control HEK 293 cells transfected with empty pcDNA3.1 vector did not show staining for RL-TGR, indicating that the α-RL-TGR antibody is binding specifically (Figure 7).

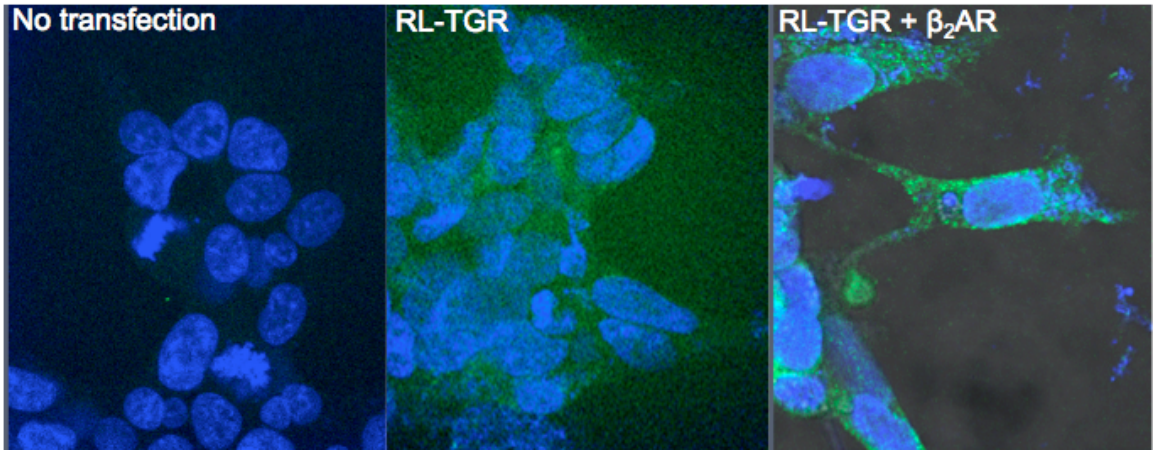

Figure 7. HEK 293 cells expressing RL-TGR. Confocal microscopy images of HEK293 cell transfected with coding region of RL-TGR and β2AR. Cells were stained for presence of RL-TGR with α-RL-TGR antibody Alexa 488 (green). The nucleus was stained with DAPI in Vectashield (blue). No transfection: HEK392 cells transfected with empty pcDNA3.1 vector. RL-TGR: HEK293 cells transfected with RL-TGR in pcDNA3.1 vector but not with β_2AR to serve as s control for trafficking to the cell membrane. RL-TGR + β_2AR: HEK293 cells transfected with RL-TGR in pcDNA3.1 and β_2AR in pcDNA3.1 vectors in the ratio of 3:1.

Since RL-TGR is known to play an important role in sensing chemical cues from the environment in zebrafish and blue head wrasse, the receptor has to be expressed on the cell membrane in order to be able to activate a singling cascade (Cohen, 2010). It has previously been shown that the trafficking of RL-TGR to the plasma membrane requires the presence of a GPCR, such as β_2AR. Therefore, as can be seen in Figure 7, only the cells transfected with both RL-TGR and β_2AR showed trafficking and expression of RL-TGR on the plasma membrane.

Functional response of RL-TGR to triterpene glycosides

To assess the scope of RL-TGR and triterpene glycoside-based chemical defenses, the functional response of RL-TGR to triterpene glycosides formoside and thornasteroside A was investigated. The functional response of RL-TGR to triterpene glycosides was measured using the cAMP assay as shown in Figure 8. HEK 293 cells expressing RL-TGR on the cell membrane were treated with triterpene glycosides, and the functional response of RL-TGR was determined by measuring corresponding cAMP levels in the cell (Figure 8). RL-TGR response to triterpene glycosides initiates a signaling cascade that results in the increased production of cAMP in the cell.

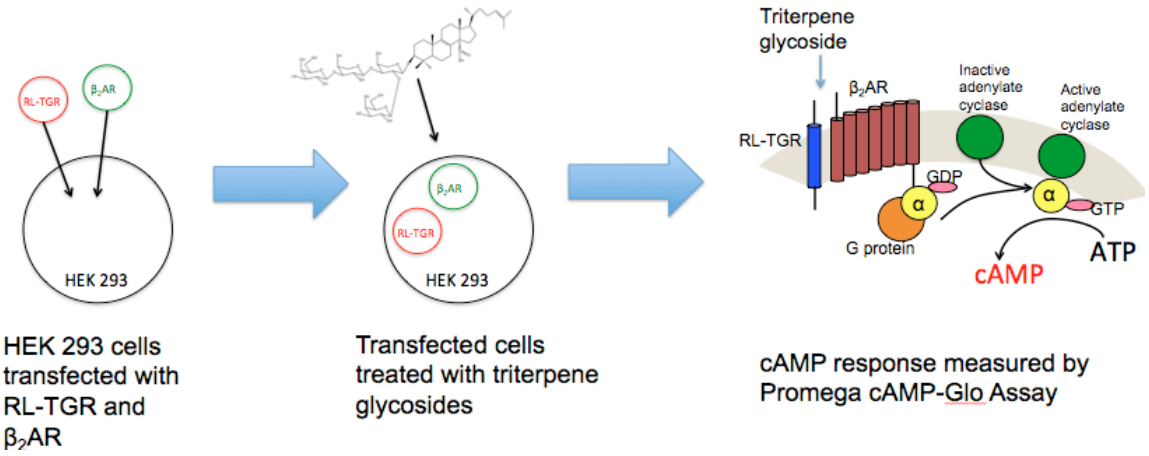

Figure 8. Process of measuring the functional response of RL-TGR to triterpene glycoside chemical defenses. HEK 293 cells were transfected with both RL-TGR and β_2AR and treated with either formoside of thornasteroside A. RL-TGR response to triterpene glycosides initiates a signaling cascade that results in the increased production of cAMP in the cell.

The cAMP assay was also performed on HEK 293 cells expressing the empty pcDNA3.1 as a negative control, and HEK 293 cells expressing only β_2AR as a positive control. It is known that isoproterenol is an agonist of β_2AR. Therefore, cells expressing

β_2AR treated with isoproterenol were expected to show increased levels of cAMP. On the other hand, cells transfected with the empty pcDNA3.1 vector were expected to show no response to treatment with either isoproterenol or triterpene glycosides.

Figure 9 shows the response of transfected HEK 293 cells when treated with 10μM of isoproterenol for 15 min. Compared to the negative control, there is a significant difference in the increase of delta RLU luminescence values in cells transfected with β_2AR only as well as in cells transfected with RL-TGR + β_2AR. Higher delta RLU luminescence values correspond to a higher level of cAMP in the cell, and therefore signify a positive response to the treatment compound. A significant increase in cAMP levels in cells expressing β_2AR compared to the negative control was observed during treatment with isoproterenol (Figure 9). Since isoproterenol is a known agonist of β_2AR, these results confirm the validity of the cAMP assay.

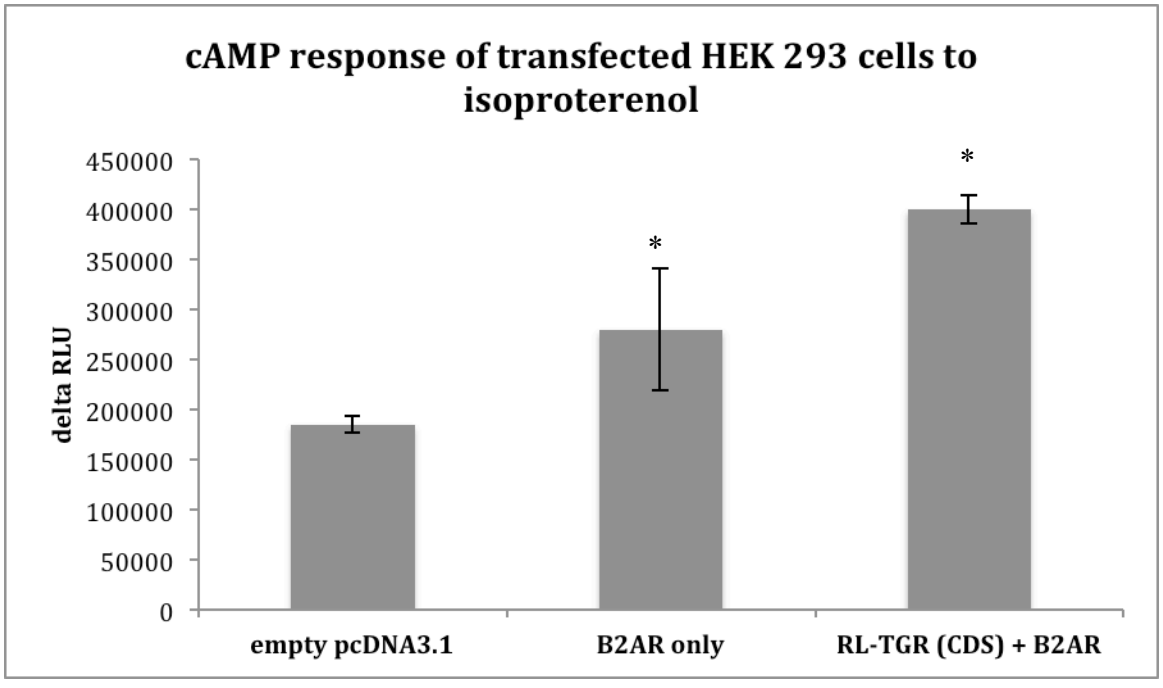

Figure 9. cAMP response of transfected HEK 293 cells to isoproterenol. HEK 293 cells transfected with empty pcDNA 3.1, with β_2AR only, and with RL-TGR and β_2AR were treated with 10μM of

isoproterenol for 15 min. Higher delta RLU values correspond to a higher level of cAMP in the cell. A paired t-test with α=0.05 was used to compare the negative control (empty pcDNA3.1) with other experimental groups, where * indicates significance (p-value < 0.05).

The response of RL-TGR to formoside was assessed by treating transfected HEK 293 cells with 10μM of formoside for 15 min. As can be seen in Figure 10, there was a significant difference in the cAMP levels in cells expressing RL-TGR + β_2AR compared to the negative control (empty pcDNA3.1). However, this difference is negative, suggesting that there was no increase in cAMP production in cells expressing RL-TGR in response to formoside. This indicates that formoside does not cause a functional response of RL-TGR. These results are surprising as formoside was previously shown to elicit a functional response of RL-TGR in electrophysiology assays (Cohen et al. 2010).

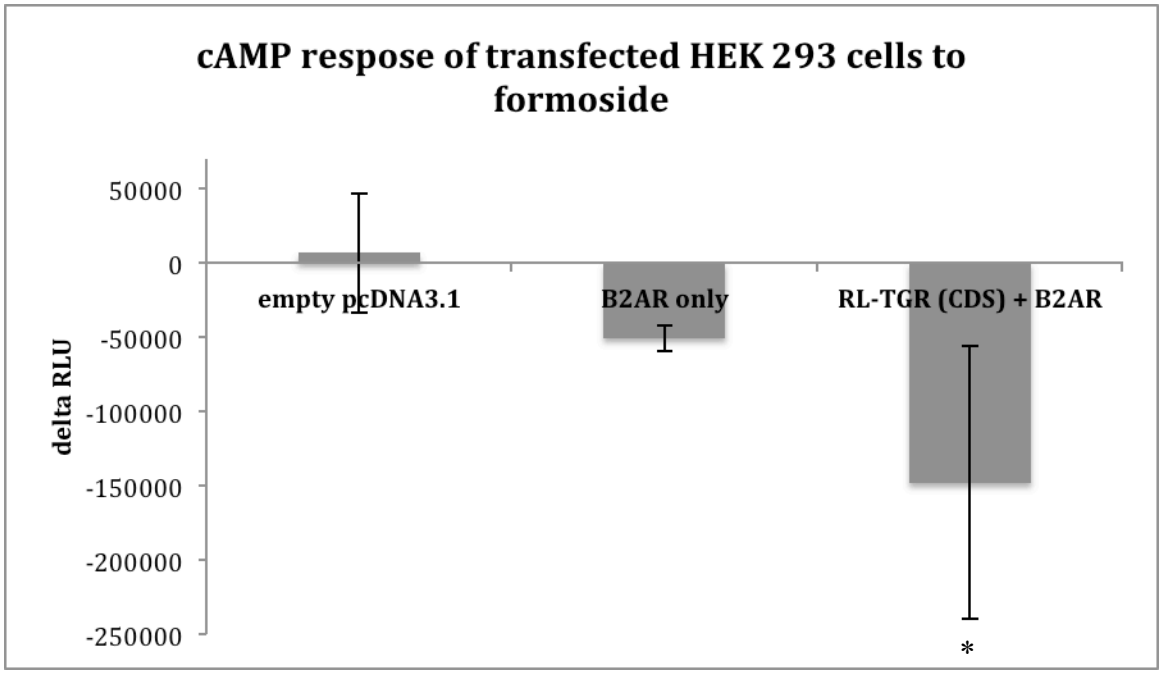

Figure 10. cAMP response of transfected HEK 293 cells to formoside. HEK 293 cells transfected with empty pcDNA 3.1, with β_2AR only, and with RL-TGR and β_2AR were treated with 10μM of formoside for 15 min. Higher delta RLU values correspond to a higher level of cAMP in the cell. A paired t-test with α=0.05 was used to compare the negative control (empty pcDNA3.1) with other experimental groups, where * indicates significance (p-value < 0.05).

To determine the scope of function of RL-TGR and triterpene glycoside chemical defenses, the response of RL-TGR to thornasteroside A was investigated. HEK 293 cells were treated with 10μM of thornasteroside A for 15 min. As can be seen in Figure 11, there is no significant difference in the cAMP levels in cells expressing RL-TGR + β_2AR compared to the negative control (empty pcDNA3.1). This signifies that thornasteroside A does not cause a functional response of RL-TGR.

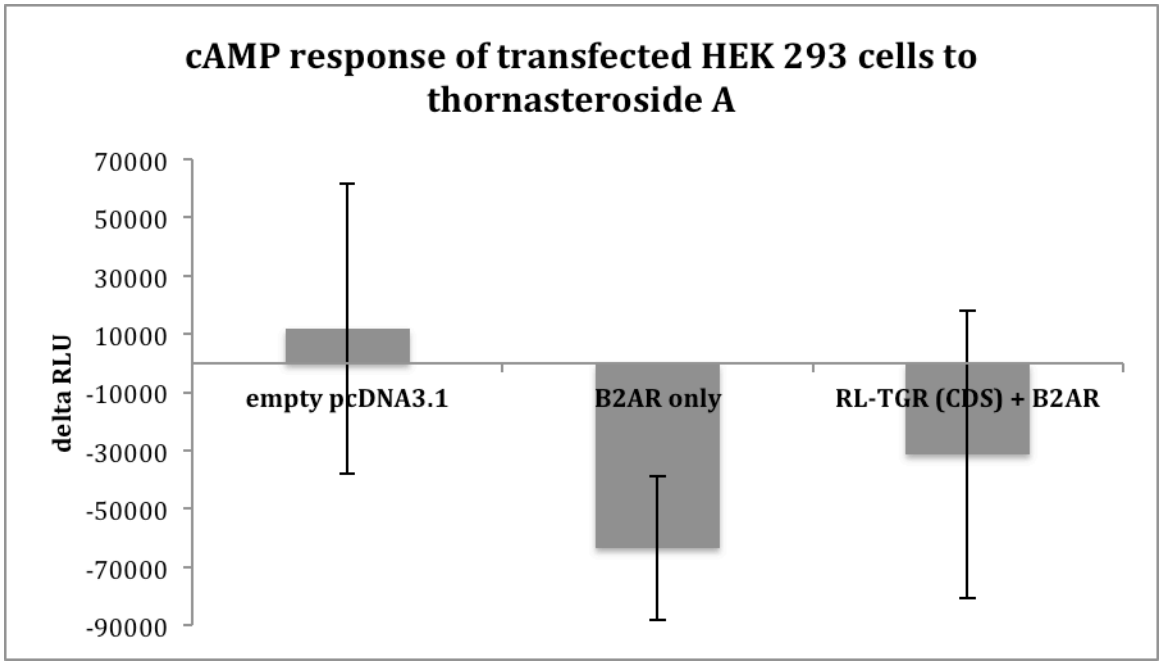

Figure 11. cAMP response of transfected HEK 293 cells to thornasteroside A. HEK 293 cells transfected with empty pcDNA 3.1, with β_2AR only, and with RL-TGR and β_2AR were treated with 10μM of thornasteroside A for 15 min. Higher delta RLU vales correspond to a higher level of cAMP in the cell. A paired t-test with α=0.05 was used to compare the negative control (empty pcDNA3.1) with other experimental groups, where * indicates significance (p-value < 0.05).

Previous studies have shown that RL-TGR responds to triterpene glycoside chemical defenses from terpenoid pathways such as formoside from *E. formosus* (Cohen

et el., 2010). The results of this study find no response of RL-TGR to formoside, which is inconsistent with previous findings. This study also finds no response of RL-TGR to a different triterpene glycoside thornasteroside A. However, this result is not very surprising as it was previously shown that not all triterpene glycosides activated RL-TGR. For example, plant derived ouabain and digoxin showed no effect on RL-TGR (Cohen et al., 2010).

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

Many marine organisms protect from predation by concentrating aversive triterpene glycosides in their tissue. It has been proposed that predatory fish response to formoside and other triterpene glycosides is mediated through chemoreception (Kubanek et al. 2000). RL-TGR, a co-receptor involved in triterpene glycoside signaling has been identified in zebrafish (Cohen et al. 2010). However, the mechanism and scope of function of RL-TGR is not fully understood. Triterpene glycosides formoside found in sea sponge *E. formosus* and thornasteroside A found in sea star *A. planci* were isolated and characterized using HPLC and ^{1}H NMR spectroscopy. The functional response of RL-TGR to these compounds was tested by expressing RL-TGR along with β_2AR in HEK 293 cells and measuring changes in cAMP levels when treated with formoside and thornasteroside A. This study found no functional response of RL-TGR to either formoside or thornasteroside A.

In the future, the cAMP assay testing the functional response of RL-TGR should be repeated to confirm the response of RL-TGR to formoside, as these results do not align with previous findings. To better understand the scope and function of RL-TGR in response to triterpene glycosides, a wider range of compounds should be tested. To this end, different triterpene glycosides from marine organisms need to be purified. In particular, the compound nodososide should be purified from sea star *L. laevigata* and tested for eliciting a response of RL-TGR. Although RL-TGR in known to respond to

some triterpene glycoside chemical defenses, the molecular mechanisms of this interaction are not fully understood. In the future, the molecular mechanisms of RL-TGR should be investigated by determining the functional domain of RL-TGR.

Chemical defenses have an important role in predator prey interactions, organization of communities and feeding behaviors in marine environments. Understanding the mechanisms of these interactions and the scope of chemical defenses in marine environments will help us understand both the chemical ecology and behavioral ecology of marine environments.

REFERENCES

Blunt, J.W., Copp, B.R., Munro, M.H., Northcote, P.T. & Prinsep, M.R. (2011). Marine Natural Products. *Natural Product Reports,* Vol.28, No.2, pp. 196–268, ISSN 0265- 0568

Cohen, S. A., Hatt, H., Kubanek, J., & McCarty, N. A. (2008). Reconstitution of a chemical defense signaling pathway in a heterologous system. *J Exp Biol, 211*(Pt 4), 599-605. doi: 10.1242/jeb.009225

Cohen SP, H. K., Halstead-Nussloch GE, Bernard KF, Hatt H, Kubanek J, McCarty NA. (2010). Identification of RL-TGR, a coreceptor involved in aversive chemical signaling. *Proceedings of the National Academy of Sciences, 107*(27), 12339-12344.

Derby, C. D., & Sorensen, P. W. (2008). Neural processing, perception, and behavioral responses to natural chemical stimuli by fish and crustaceans. *J Chem Ecol, 34*(7), 898-914. doi: 10.1007/s10886-008-9489-0

Kubanek J, P. J., Eve ™, Fenical W. (2000). Triterpene glycosides defend the Caribbean reef sponge Erylus formosus from predatory fishes. *Marine Ecology Progress Series, 207*, 69-77.

Kubanek, J., Whalen, K., Engel, S., Kelly, S., Henkel, T., Fenical, W., & Pawlik, J. (2002). Multiple defensive roles for triterpene glycosides from two Caribbean sponges. *Oecologia, 131*(1), 125-136. doi: 10.1007/s00442-001-0853-9

Lindsay, S. M. (2004). Behavioral Responses of Newly Hatched Zebrafish (Danio rerio) to Amino Acid Chemostimulants. *Chemical Senses, 29*(2), 93-100. doi: 10.1093/chemse/bjh009

Lunceford, B. E., & Kubanek, J. (2015). Reception of Aversive Taste. *Integr Comp Biol, 55*(3), 507-517. doi: 10.1093/icb/icv058

Pawlik, J., Chanas B. (1995). Defense of Carribean sponges against predatory reef fish. *Mar Ecol Prog, 127*, 195-211.